



Hawaii Biotechnology Group, Inc.

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October 31, 1994

Re: Contract Number N00014-93-C-0019

Commanding Officer
Naval Medical Research and Development Command
Scientific Officer: LT CDR P. Knechtges
National Naval Medical Center, Bldg. 1, 12th Floor
8901 Wisconsin Avenue
Bethesda, MD 20889-5606

Dear Lt. Commander Knechtges:

Enclosed is the progress report for the seventh quarter (7/1/94-9/30/94) of the contract period. The accompanying report describes our current progress for each portion of the contract. If you have any questions, please do not hesitate to contact me (ext. 399).

Sincerely,

Steven A. Ogata, Ph.D.
Scientist

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I. Overview

The following progress report covers the seventh quarter (7/1/94-9/30/94) for contract number N00014-93-C-0019. Four topics are included in the contract; each topic's goal and current status are as follows:

1. Production of anti-ferret sIgA antibodies. Status: Rabbit immunization completed. Immune serum collected. Cross-adsorbing rabbit serum and mouse ascites.
2. Purification of lipopolysaccharide from *Shigella* and *Campylobacter* species. Status: *Shigella* LPS delivered to Navy. Collecting *Campylobacter jejuni* 81-176 cells for LPS extraction. Extraction method identified. Exploring alternative sources of *Campylobacter* LPS.
3. Development of an enzyme immunoassay for the detection of enteroaggregative *Escherichia coli* heat-stable toxin. Status: This portion of the project has been suspended.
4. Production of monoclonal antibodies against strain-specific antigenic epitopes on *Campylobacter coli* flagella. Status: Completed third fusion. No strain-specific MAbs identified. Discussing changes in protocol with NMRI researchers.

II. Current Progress

1. Production of Anti-ferret sIgA Antibodies

Production of rabbit α -ferret sIgA. During the past quarter, we received ferret serum from Naval researchers and purified serum IgG by affinity chromatography on Staph Protein A sepharose. The purified IgG was conjugated to an Affi-prep 10 (Bio-Rad, Hercules, CA) support for cross-adsorption of polyclonal rabbit α -ferret sIgA (RAFS) antisera. Our initial column contained 6mg of purified ferret IgG on 2ml of Affi-prep matrix. Cross-adsorption of 1ml of RAFS antisera (diluted 1/35 in column buffer) by circulation through the ferret IgG-Affi-prep column did not reduce cross-reactivity against ferret IgG on Western blots of clarified ferret milk.

Regeneration of the ferret IgG-Affi-prep column by elution with citrate buffer, pH 3, released two components as determined by SDS-PAGE: a minor band with the same relative mobility as ferret IgG and a major band with relative mobility of ~ 150 kD. In SDS-PAGE, both

mouse and ferret IgG have relative mobilities of $\sim 200\text{kD}$; therefore, we believed that the major component was not rabbit IgG and hypothesized that it was a subunit of the complement cascade component C1q, which is capable of binding to the F_c portion of IgG. To determine the identity of the major component, we purified RAFS IgG by affinity chromatography on Staph Protein A sepharose and examined it by SDS-PAGE. The purified rabbit IgG had a relative mobility of $\sim 150\text{kD}$, thus indicating that the major component eluted from the ferret IgG-Affi-prep column could be rabbit IgG. Western blot analysis using goat α -rabbit IgG verified the identity of the eluted material as rabbit IgG. Based upon the absorbance at 280nm (Abs_{280}) of the eluted rabbit IgG, at least 2mg had bound to the ferret IgG-Affi-prep column [approximately one-fifth of the estimated amount of IgG (10mg; Harlow and Lane, 1988) in the starting sample].

Purified IgG from RAFS antisera was used for subsequent cross-adsorption experiments, since we felt that removal of other immunoglobulin isotypes, that may competitively bind to the column, would improve adsorption of cross-reactive IgG. Unexpectedly, we discovered that the purified IgG did not bind to the regenerated column. As it was possible that the IgG had been denatured during purification, we determined its reactive titer (based upon IgG content) on Western blots of clarified ferret milk and found a reduction of ~ 2 -fold relative to that of the RAFS antisera. Whereas this reduction would reduce binding, it would not account for the absence of binding that was encountered.

To determine whether the regeneration of the ferret IgG-Affi-prep column by acid elution had not restored its functionality, we first analyzed the regenerated column matrix by SDS-PAGE and found rabbit IgG was still bound, thus indicating that binding sites on the ferret IgG may still be blocked. We then batch incubated 0.25ml of column matrix with RAFS antisera ($\sim 500\mu\text{g}$ of IgG; reaction mixture volume $\sim 0.5\text{ml}$) to determine whether the column had been destroyed. Seventy four micrograms of rabbit IgG was eluted from the column matrix, thus indicating that the column was still functional. Based upon our previous results, the binding capacity of 0.25ml of column matrix was at least $250\mu\text{g}$ of ferret IgG, thus the binding capacity had been reduced. When we batch incubated $500\mu\text{g}$ of purified IgG from RAFS antisera with another 0.25ml aliquot of regenerated column matrix (reaction volume $\sim 1\text{ml}$), purified IgG bound to the column. Approximately one-fourth the amount of IgG determined for RAFS antisera was eluted. Taking into consideration that the purified IgG possessed one-half the reactive titer of RAFS antisera and was incubated with the matrix at one-half the concentration, the efficiency of binding was equivalent to that of RAFS antisera.

Since our results using the regenerated matrix were conflicting, we prepared an additional 2ml of ferret IgG-Affi-prep (9mg IgG bound to 2ml Affi-prep), combined it with the regenerated matrix, and cross-adsorbed 3mg of purified IgG from RAFS antisera by batch incubation (0.7mg IgG per ml reaction mix). Although Abs_{280} readings indicated that purified IgG was not bound

to the column, Western blot analysis indicated that a reduction in reactivity against intact ferret IgG had occurred. Significant reductions in reactivities against a number of lower molecular weight bands, however, were not observed. These same bands reacted with goat α -ferret IgG (not γ -specific); therefore, we believe that they are proteolytic cleavage products of ferret immunoglobulins. The presence of antibodies that recognize immunoglobulin degradation products could produce erroneous results when determining total sIgA content (provided immunoglobulin degradation products are present in test samples) or α -*Campylobacter* sIgA titers (provided non-sIgA degradation products can bind to *Campylobacter* antigens); therefore, cross-adsorption of RAFS antisera with ferret IgG-Affi-prep alone will be inadequate to prepare a satisfactory product..

Cross-adsorption of mouse ascites. We had previously reported that murine ascites fluid from mice immunized with purified ferret sIgA was efficiently cross-adsorbed by batch incubation with ferret IgG-Affi-gel 10 matrix (see First Year Report). Since cross-adsorption of the RAFS antisera is proving to be difficult, we decided to cross-adsorb some of the ascites for use by Naval researchers while work on the rabbit antisera continued. Repeated cross-adsorption of an aliquot of ascites fluid with ferret IgG-Affi-prep reduced cross-reactivity against intact ferret IgG; however, reactivity against immunoglobulin degradation products was not affected. Upon review of our previous experimental results, it was apparent that reactivity against these components had been reduced but not removed by cross-adsorption. This is in contrast to cross-adsorption of mouse α -ferret sIgA antisera by incubation with unbound ferret IgG which effectively removed reactivity against all ferret milk components other than intact sIgA. Based upon these results, it is reasonable to conclude that covalent linkage of the IgG to a solid matrix is affecting cross-adsorption.

In addition to reducing cross-reactivity against intact ferret IgG, cross-adsorption of the mouse ascites with ferret IgG-Affi-prep reduced reactivity against ferret sIgA ~40-fold. This was unexpected as reductions of 4-8 fold had been encountered in previous cross-adsorptions. We believe that the increased reduction was due to the presence of contaminant sIgA in our current ferret IgG preparations. For earlier preparations, ferret IgG was isolated by affinity chromatography on Staph Protein A sepharose, then further purified by anion-exchange chromatography on DEAE sepharose to remove contaminant sIgA. Based upon SDS-PAGE analysis of recent ferret IgG preparations, we felt that the amount of contaminant sIgA would have negligible effect upon the α -sIgA reactivity; however, our cross-adsorption results suggest that this is not the case. To verify that contaminant sIgA is the problem, we will purify ferret IgG using both affinity and anion-exchange chromatography. The purified IgG will be conjugated to Affi-prep 10, and the ferret IgG-Affi-prep matrix will be used to cross-adsorb ascites. If contaminant sIgA is the cause of the reduction, cross-adsorption with the new matrix should not result in a 40-fold reduction in α -sIgA activity.

Since cross-adsorption with ferret IgG bound to Affi-prep did not remove reactivity against immunoglobulin degradation products, we cross-adsorbed the ascites using Western blots of clarified ferret milk. Prior to adsorption, the sIgA portion of the blot was removed to avoid reduction of sIgA-specific reactivity. Cross-adsorption by this method significantly reduced reactivity against immunoglobulin degradation products without reducing reactivity against sIgA. Thus, the antibodies that recognize the degradation products do not react, or react weakly, with the intact sIgA molecule. Using nitrocellulose-bound milk components, however, is not a feasible method for cross-adsorption of large quantities of antisera. For this reason, development of affinity columns containing either ferret sIgA (preferable method) or ferret milk components other than sIgA will be necessary. If the former is to be prepared, we must identify a source of ferret sIgA with a greater sIgA content than our current ferret milk stock which contains an estimated 40 μ g per ml (based upon sIgA purification yields).

2. Extraction and Purification of Lipopolysaccharide

During the seventh quarter, we identified a LPS purification method with a 12-fold greater yield than that achieved using the phenol-water method. The protocol was developed by Perez and Blaser (1985) for the extraction of LPS from *Campylobacter* species and utilizes the detergent sodium dodecyl sulfate (SDS) to solubilize the LPS. Using this method, we increased our yield from 0.05% (see Sixth Quarter Report) to 0.6% LPS per estimated dry weight of cells (assuming 70% cell water content). Analysis of the purified LPS did not detect contaminant protein nor nucleic acids.

In the Sixth Quarter Report, we reported poor yields of *C. jejuni* 81-176 cell mass from biphasic cultures. During the seventh quarter, we compared cell mass yields from cultivation of *C. jejuni* 81-176 on Mueller-Hinton agar (48 hours, 37°C, microaerophilic conditions) with that of biphasic media (48 hours, 37°C, aerobic conditions). Based upon our results, we can collect the equivalent cell mass of a 500ml biphasic culture (400ml broth overlay, 100ml agar base) from 4 agar plates. Our cultivation capacity, however, is limited as the GasPak container, that is used to maintain the microaerophilic conditions, can hold only 36 (100x15mm) plates. Based upon our yields from the SDS-extraction method, we would need to collect growth from 2,640 plates (264g wet weight of cells) to produce 200mg of LPS. This would require 73 cultivations and 36-37 weeks.

For the production of 200mg of *C. jejuni* 81-176 LPS to be feasible, either the extraction efficiency must be increased or an alternative source of crude LPS must be identified. For this reason, we explored the possibility that *C. jejuni* 81-176 secretes LPS in biphasic cultures. This study was prompted by an article by Gu and Tsai (1991) in which they reported purifying LPS from outer membrane vesicles (OMV's) secreted by *Neisseria meningitidis* in broth culture.

Comparison of LPS from *N. meningitidis* OMV's and cells from 1ml of broth culture revealed approximately 2.5 times as much LPS in OMV's as in cells (47.9 μ g vs 18.5 μ g). The LPS from the two sources were indistinguishable by SDS-PAGE and produced similar results when tested in the *Limulus* amoebocyte lysate assay. Should *C. jejuni* 81-176 secrete similar amounts of LPS, it may be possible to collect the required 200mg from culture supernatants. At present, our preliminary results suggest that LPS is present in the culture supernatants; however, further experimentation is necessary.

3. Enteroaggregative *Escherichia coli* Heat-Stable Toxin 1 (EAST1)

Captain Bourgeois has informed us that Dr. Savarino has not been able to create recombinants that are satisfactory for our purposes. As financial funds are a major limitation, this task has been essentially terminated, thus further work was not performed during the past quarter.

4. Type-Specific *Campylobacter* Flagellin Epitopes

Production of α -Flagellin Monoclonal Antibodies. We have completed subcloning chosen hybridomas from the three fusions. Hybridomas that produce T2-specific MAbs have not been isolated from any of the fusions. We have also tested some of the MAbs on Western blots of whole cell lysates of *Campylobacter coli* VC167 T1, *C. coli* VC167 T2, *C. coli* VC167 T2316-7A, and *C. jejuni* 81-176. At present, we have tested MAbs produced by 7 of the 17 hybridomas that have been frozen and anticipate testing the remaining 10 in the near future. Of the seven that have been tested, 3 react with flagellin and other bands, 2 react with bands other than flagellin, and 2 have no visible reactivity.

Additional work regarding the effect of MAb CCB 227 upon invasion of host cells by *C. coli* VC167 T2 was not performed, since the production of LPS and cross-adsorption of rabbit α -ferret sIgA are of higher priority. When time permits, we intend to continue the invasion studies.

III. Plans for Present Quarter

Due to difficulties initially encountered for the EAST-1 portion of the contract, the probability of completing this task is low. For this reason, we were to discuss the possibility of refocusing the project emphasis with Captain Bourgeois of NMRI. During July, Dr. Ogata was to attend a Naval workshop in Egypt at which time he could discuss the possible reorganization with Captain Bourgeois and other NMRI researchers; however, due to illness, he could not attend the meeting. A second meeting was to be held in Maryland and coordinated by Captain Bourgeois; however, due to illness in his family, he was unable to arrange the trip. At present,

discussions on reorganizing the project have been terminated.

The interruption in funding that occurred in the middle of the grant period and subsequent, temporary suspension of specific tasks has made it unlikely that we will complete the goals of this contract within the specified period. Due to the availability of adequate funds for continued work, we will request a no-cost extension through July 1995.

1. Production of Anti-Ferret sIgA Antibodies

We intend to purify more ferret serum IgG by affinity and anion-exchange chromatography and couple the purified IgG to Affi-prep 10. The ferret IgG-Affi-prep, along with Western blots of ferret milk, will be used to cross-adsorb mouse ascites containing α -ferret sIgA antibodies. We will supply the cross-adsorbed ascites to Naval researchers for use, while we continue our efforts to cross-adsorb the rabbit antisera.

2. Extraction and Purification of Lipopolysaccharide

As of this writing, we have modified the SDS-extraction method and increased the LPS yield to ~5%. Therefore, during the current quarter, we will cultivate, collect, and extract *C. jejuni* 81-176 cells to produce the specified 200mg of LPS. In addition, we will complete the examination of supernatants from broth cultures of *C. jejuni* 81-176 to establish the presence of LPS and determine its potential as a source of LPS.

3. EAST1

No work planned due to suspension of this portion of the project.

4. Type-Specific Campylobacter Flagellin Epitopes

We intend to begin immunizing mice for future fusions following discussion with NMRI researchers regarding changes in protocol.

IV. References

- Gu, X-X, and C-M Tsai. 1991. Purification of rough-type lipopolysaccharides of *Neisseria meningitidis* from cells and outer membrane vesicles in spent media. *Anal. Biochem.* 196:311-318.
- Harlow, E., and D. Lane. Antibodies. A laboratory manual. Cold Spring Harbor laboratory. Cold Spring Harbor, New York. 1988, p291.
- Perez, G.I.P., and M.J. Blaser. 1985. Lipopolysaccharide characteristics of pathogenic campylobacters. *Infect. Immun.* 47:353-359.